

Final Report

Project Title: Role of microbial synergies in immobilization of metals

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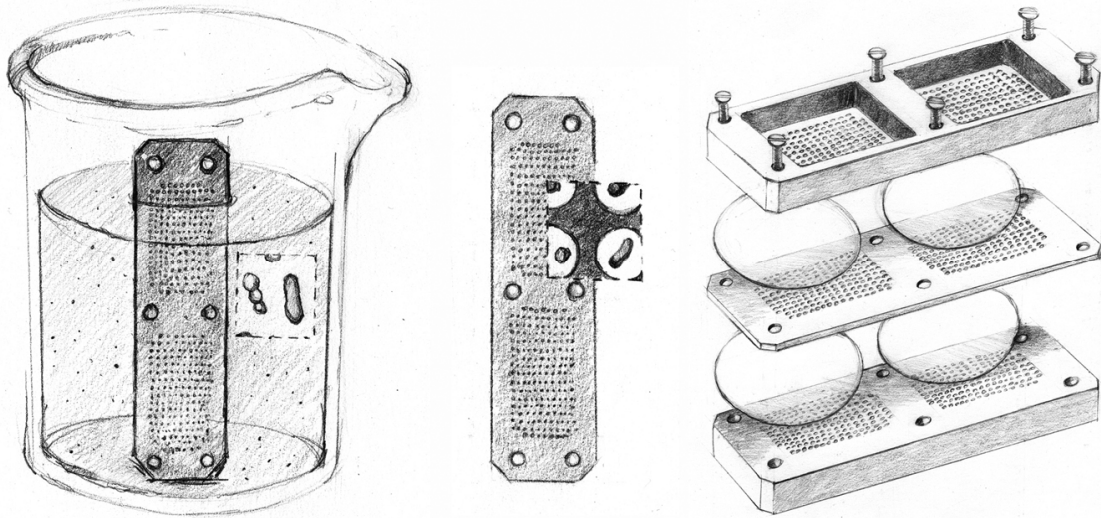
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This project aimed at cultivating previously uncultivated microorganisms and their consortia that are involved into metal immobilization in groundwater at selected FRC sites. The principle objectives were to cultivate the main microbial players in the target environment, determine their rates of metal immobilization, and investigate whether the presence of synergistic species enhanced the rate of this immobilization. The objectives have not changed during the course of the study. The principle developments and findings of this research are as follows.

1. For cultivation/isolation/domestication studies, we used a well-developed principle of *in situ* incubation of target microorganisms inside diffusion chambers. However, the specific design of the diffusion chambers needed optimization for the purposes of this specific project. The originally proposed Teflon-coated steel chips (OpenArray™ Plates by BioTrove, Inc) are excellent for microbial isolation, but their material could potentially interfere with fine analytical tools used to detect heavy metal precipitation. Our plan was to explore Teflon plates as a substitute for the OpenArray™ plates. Upon extensive testing, however, we discovered that these (Teflon-based) plates were lacking rigidity necessary to attach membranes to the plates in order to seal the inner environment of the chamber. As a result, contamination became a recurrent issue. We spent considerable time and effort looking for a good substitute for the above (OpenArray™ and Teflon) chips, and in parallel changed the design of the chambers to make isolation more efficient. Following suggestions of material scientists from Argonne National Laboratory, we have successfully identified such material, Delrin. One of Argonne's subcontractors, HiTech Manufacturing, Inc., manufactured several devices for diffusion-based microbial cultivation, hereafter referred to as isolation chips, or ichips for short.

The ichip consists of three plates with matching holes, as illustrated on the picture below. The middle plate is dipped into a cell suspension and then removed; the through-holes sample the suspension in the form of tiny droplets. Then 0.03-um membranes are placed over both sides of this plate, and the remaining two plates with matching holes are placed over the membranes. The ichip is thus a 5-layer sandwich held together by screws, which, once tightened, press the membranes against the central plate sealing

the contents of the individual holes. The ichip is then incubated in the natural environment, which provides the cells inside the miniature diffusion chambers with the natural suit of nutrients and growth factors.



We have conducted extensive tests of the ichips on their ability to grow and isolate environmental microorganisms in both soils and water. We are very happy with the performance of the ichips, especially since removal of grown material is very easy. Delrin is a fairly hydrophobic material, and agar plugs with growing colonies can be easily removed just by touching these plugs, as they readily fall out (into e.g. a well of a 96-well plate, or Eppendorf tube). The amount of agar in each miniature chamber is minimal (around 1 microliter), which allows for quality microscopy observations of grown material prior to removal/isolation. The number of holes/diffusion chambers is over 350 per plate, which allows massively parallel isolation. The size of the ichip is 3 x 1 x 0.5 inch, and so they can be lowered into, incubated in, and removed from even the thinnest boreholes at the planned FRC field sites. We published a paper describing this new technology which attracted a significant amount of interest among microbiologists (Nichols, D., Cahoon, N., Trakhtenberg, E.M., Pham, L, Mehta, A., Belanger, A., Kanigan, T., Lewis, K., and Epstein, S.S. (2010). Ichip for high-throughput in situ cultivation of “uncultivable” microbial species. *Appl. Environ. Microbiol.*, 76: 2445-2450)

2. We have deployed ichips multiple times, at both FRC Sites 2 and 3, in boreholes FW106 and GW835, respectively. Prior to the deployment, we sampled the groundwater material for microbial enumeration. DAPI-stained preparations suggested the microbial abundance of 10^5 cells/ml. Field samples were diluted in warm agar so as provide each through hole of the ichips with 5-20 cells.

Predictably, all agar plugs in the through holes showed microbial growth. The plugs were removed, their contents syringe mixed, and subcultured on 1.5% agar supplemented with 0,01% casein, casaminoacids, and yeast extract. Almost 20% of cultures from Site 2, and over 25% of cultures from Site 3 subsequently grew, resulting in 288 and 384 microbial consortia growing *in vitro*, respectively. The 672 microbial consortia obtained were deemed sufficient to begin their screening on the ability to reduce metals of interested.

3. In parallel with designing and testing of new incubation devices, we worked on details of analytical experiments. The originally proposed technique, Laser-Induced Breakdown Spectroscopy, proved not practical after Dr. Madhavi Martin, our original collaborator from ORNL, left the lab. This, and the extensive search for the optimal alternative, caused rescheduling the analytical part of our project. We successfully identified such an alternative - Inductively Coupled Plasma Mass Spectrometry, or ICP-MS, and our ORNL collaborators Drs. Palumbi and Yang provided convenient access to the related instrumentation at the ORNL.

ICP-MS is an excellent method for our study because it provides a fast, cost-efficient, and simultaneous determination of the majority of elements in the periodic table. The detection limit for most of the elements of interest is below one part per trillion, far below our actual needs; the rest can be quantified starting from 1 to 10 ppt, which is also more than sufficient. We worked out all the necessary details of sample preparation at Northeastern University meeting the demands of ICP-MS, and conducted several rounds of calibration experiments using the original isolates from the FRC sites as well the positive control microorganisms, *Shewanella* strain CN32, provided to us by Dr. Yang. These experiments concerned quantification of uranium and microbe-mediated reduction.

4. We screened the 672 microbial consortia isolated in item 2 on their ability to reduce uranium. The mixes of species first grown in the ichip *in vivo* were maintained *in vitro* as described in Item 2. For metal reduction experiments, their biomass was scaled up to approximately $10^8 - 10^9$ cells/ml, and a portion of the culture was then inoculated into the fresh medium containing uranyl acetate at 3, 30, and 300 ppb. These were incubated anaerobically for 7-19 days, and the cultures were filtered through 0.2-um pore-size filters. This separated cell biomass, and thus uranium in its (insoluble) reduced form, from the solution with unmodified uranyl acetate. Uranium in the cell material is then oxidized by nitric acid and quantified by ICM-MC; uranium in solution is also quantified to measure the portion of the initial amount that had not been converted by microorganisms into the insoluble form. Media with no uranium acetate, with and without heat-killed cells, served as negative controls; *Shewanella* CN32 served as a positive control.

The screens indicated that approximately 2/3 of our consortia were capable of reducing uranium, however, none at the rate exceeding that characteristic of *Shewanella* CN32.

5. Our Objectives called for identification of microbial consortia that perform metal immobilization better than do the individual species. We identified 10 microbial consortia exhibiting the highest rate of uranium reduction. We then successfully dissected 6 of them and isolated individual species comprising these consortia. Most species proved incapable of uranium reduction as detected by the analytical methods used but each consortium contained 1-2 species that did reduce it. The rate of the reduction were statistically indistinguishable from that of the original consortia and were equal to or below the positive control (*Shewanella* CN32). We concluded that the microbial partners in the consortia examined did not increase the rate of uranium reduction relative to that characteristic of individual species.

6. Several isolates we obtained stood out as particularly novel. Phylogenetic analysis of 16S rRNA gene sequences revealed clear differences between diversity of isolates

obtained by the diffusion chamber approach and those obtained by conventional plating. The latter approach led to isolation of Alpha- and Gamma-Proteobacteria, Actinobacteria and Verrucomicrobia. Isolates obtained via the diffusion chamber approach represented Alpha-, Beta-, and Gamma-Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes. Notably, one third of the isolates obtained by the new method were closely related to species known from previous molecular surveys conducted in the FRC area. Since the initial growth of microorganisms inside diffusion chambers occurred in the presence of the environmental stress factors, we expected the isolates we obtained to be tolerant of these factors. We investigated physiology of selected isolates and discovered that their majority were indeed capable of growth under low pH and/or high concentrations of heavy metals and nitrate. This indicated that, in contrast to conventional isolation, the diffusion chamber-based approach leads to isolation of species that are novel, exhibit tolerance to extant environmental conditions, and match some of the species previously discovered by molecular methods. A paper with high citation index was published on the basis of these results (Bollmann A, Palumbo A.V., Lewis K., and Epstein, S.S. (2010). Isolation and physiology of bacteria from contaminated subsurface sediments. *Appl. Environ. Microbiol.* 76: 7413–7419)

7. Several isolates representing Bacteria and Fungi proved particularly resistant to all toxic factors present in the groundwater at FRC. In fact, several grew well at concentrations of several pollutants (Cd, U) exceeding those measured in situ. We are in the process of sequencing their genomes. The genome of one isolate has been finished and reported (Brown, S.D., Palumbo, A.V., Panikov, N., Ariyawansa, T., Klingeman, D.M., Johnson, C.M., Land, M.L., Utturkar, S.M., S.S. Epstein (2012). Draft Genome Sequence for *Microbacterium laevaniformans* Strain OR221, a Bacterium Tolerant to Metals, Nitrate, and Low pH. *J Bacteriol* 194:3279). The genomes of two more bacterial and one fungal isolates have been annotated and are being prepared for publication. We are in the process of analyzing the genomic potential explaining the exceptionally high resistances of these microorganisms.